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MYCORRHIZA-INDUCED ALLEVIATION OF PLANT DISEASE CAUSED BY *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* AND ROLE OF ETHYLENE IN MYCORRHIZA-INDUCED RESISTANCE IN TOMATO

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The protective role of arbuscular mycorrhizal fungi (AMF) against the phytopathogen *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) was examined in tomato plants. Seven different AMF isolates were used to determine which ones were able to induce effectively resistance against Cmm. Stems of seven-week tomato plants were infected with Cmm, then a disease severity index (DSI) was determined during the next three weeks. In addition to different responses to mycorrhizal inoculation, three levels of responses to the bacterial disease were recognized in treatments. Plants inoculated with *Rhizophagus irregularis* (Ri) showed both the highest colonization and the highest induced resistance to Cmm while the effect of *Funneliformis mosseae*, *Gigaspora margarita* and *Claroideoglomus claroideum* on mycorrhizal colonization and on the induced resistance were intermediate and high, respectively. Subsequently, Ri was chosen to inoculate ethylene-insensitive tomato mutant line Never ripe (Nr) and its background (Pearson) to investigate the possible role of ethylene (ET) in the mycorrhiza-induced resistance (MIR). The results showed that Ri could induce systemic resistance against Cmm in the Pearson background, whereas ET-insensitivity in Nr plants impaired MIR. These results suggest that ET is required for Ri-induced resistance against Cmm. To our knowledge, this is the first study to examine the effect of different AMF isolates on the response of tomato plants to Cmm and involvement of ET in MIR against Cmm.

Keywords: *Clavibacter michiganensis* subsp. *michiganensis* – arbuscular mycorrhizal fungi – mycorrhiza-induced resistance – tomato – ethylene

INTRODUCTION

Tomato is an important vegetable plant cultivated under a wide range of production system throughout the world. However, tomato production worldwide incurs severe yield losses by the most important bacterial disease *Clavibacter michiganensis* subsp.

Abbreviations: AM, arbuscular mycorrhizal; AMF, arbuscular mycorrhizal fungi; Cmm, *Clavibacter michiganensis* subsp. *michiganensis*; Cc, *Claroideoglomus claroideum*; DSI, disease severity index; ET, ethylene; Fg, *Funneliformis geosporum*; Fm, *Funneliformis mosseae*; Gm, *Gigaspora margarita*; MIR, mycorrhiza-induced resistance; Sc, *Septoglomus constrictum*; Ri, *Rhizophagus irregularis*; Rs, *Rhizophagus* sp.

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michiganensis (Cmm). It is a causal agent of the wilt and canker disease [7], as well as one of quarantine organisms that are subject to international quarantine regulations. Arbuscular mycorrhizal (AM) symbiosis is the most ubiquitous mutualistic association formed between arbuscular mycorrhizal fungi (AMF) and plant roots. In addition to improved water and nutrient uptake in the host plant, AMF could enhance plant resistance against various pathogens [17]. Indeed, many studies describe that mycorrhizal colonization increases plant resistance against pathogenic fungi and bacteria [10, 24, 30, 38].

The use of arbuscular mycorrhizal fungi provides a sustainable alternative for plant disease management [21]; however, the underlying mechanism of AMF-induced disease resistance remains elusive. Most studies focused on only a single isolate or a few AMF species (mostly *Glomus intraradices* and *G. mosseae*), giving conflicting reports on the specificity of AMF. Despite evidences of AMF potential in controlling plant diseases, little attention has been paid to the effects of mycorrhization on plant resistance to Cmm. There is a only single work demonstrating a neutral effect of extraradical mycelium of *G. intraradices* on the growth of Cmm [9].

Plant defense responses highly rely on its hormones to coordinate a complex defense system to combat against pathogens. Among phytohormones, ethylene (ET) plays an essential role in the regulation of plant immunity [2], and a significant role in microbe–plant interactions [18]. During Cmm infection, ET has been proved to be crucial in the development of wilting and canker symptoms [4]. Many studies highlight that resistance against a wide range of phytopathogens induced by beneficial microbes largely depends on the Jasmonate/ET signalling pathway [15, 19, 42].

The objectives of this study were to reveal out whether AMF isolates were able to induce systemic resistance to Cmm in tomato plants, if so, a possible role of ET signalling pathway in mycorrhiza-induced resistance (MIR).

MATERIALS AND METHODS

Preparation of different AM inoculums

Mycorrhiza inoculums including seven different AMF species were propagated separately in pot cultures of *Zea mays* and *Medicago truncatula* for 9 months. The basic AMF samples, *Funneliformis mosseae* BEG 12 (Fm), *Funneliformis geosporum* BEG 11 (Fg), *Rhizophagus irregularis* MUCL43194 (DAOM197198) (Ri), *Rhizophagus* sp. MUCL43204 (Rs), *Septoglomus constrictum* (formerly *Glomus constrictum* Trappe.) (Sc), *Claroideoglomus claroideum* BEG 23 (Cc), *Gigaspora margarita* BEG 34 (Gm) originated from different national mycorrhizal collections. Mycorrhizal inoculums consisted of sand, mycelia, infected root fragments and spores. Spores of each AMF species in inoculums were examined and adjusted to 22 spores/g on average before inoculation. Thirty grams of inoculum were placed at 3 cm below pre-germinated seeds in plastic pots at the time of transferring the seeds. Non-AM plants

were received thirty grams of autoclaved mycorrhizal inoculum and 3 ml aliquot of a filtrate ($<20\ \mu\text{m}$) of the AM inoculum to supply a general microbial population free of AM propagules.

Plant material and growth conditions

Two consecutive experiments were conducted as followings.

Experiment 1: Effect of different AMF isolates on tomato plant resistance against Cmm

Tomato seeds (*Solanum lycopersicum* L. cv. MoneyMaker) were treated with 2.7% sodium hypochlorite containing 0.02% (v/v) Tween-20 for 30 min, then washed with sterilized distilled water several times, and put on the filter paper in Petri dishes at 26 °C for 3 days for germination. Pre-germinated seeds were sown in each plastic pot containing 0.5 kg of sterile sand:peat (4:1, v/v) mixture. Before planting the seeds, different mycorrhizal inoculations, representing different treatments were prepared. There were eight treatments including plants inoculated separately with one of seven different AMF isolates altogether with non-AM plants. Thirty replicates of each treatment settled in a growth chamber. Pots were randomly distributed and cultivated at 23/28 °C with 16/8 hours of photoperiod, light intensity of 600 $\mu\text{mol}/\text{m}^2/\text{s}$ and 60% humidity. Pots were watered twice and fertilized once a week with Long Ashton nutrient solution [14], adjusted to 3.2 μM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. After 7 weeks of growth, the bacterial pathogen Cmm injection was performed, as described below. When plants reached 10 weeks of growth, plant biomass, mycorrhizal colonization and disease severity index were determined.

Experiment 2: Role of ethylene in Rhizophagus irregularis-induced resistance against Cmm

Tomato seeds (*Solanum lycopersicum* L.) of Never ripe (Nr), ethylene-insensitive mutant, and its background Pearson kindly provided by Tomato Genetics Resource Center (University of California, Davis) were used. Before planting the seeds, inoculation with *Rhizophagus irregularis* (MUCL43194) and non-inoculation were implemented in each genotype. Fourteen replicates of each treatment were distributed randomly in a growth chamber. All growth conditions were the same as the description in experiment 1. Cmm injection was performed after 7 weeks of plant growth, as described below. Shoot fresh and dry weight, mycorrhizal colonization and disease severity index were examined at 10 weeks of plant growth.

Bacterial pathogen infection and measurement of disease severity index

Clavibacter michiganensis subsp. *michiganensis* (B.01778) from National Collection of Agricultural and Industrial Microorganisms (Hungary) was cultured in LB medium at 27 °C for 72 h. Bacterial suspension was concentrated by centrifugation at 5,400 g for 20 min, washed twice and diluted to 10⁹ CFU/ml using sterile 10 mM MgCl₂. An equal quantity of bacterial suspensions (50 µl) was injected into the stem region between the cotyledons of 7-week-old plants with a syringe fitted with a 30-gauge needle while 50 µl sterile 10 mM MgCl₂ solution was used for mock-infected plants (non-Cmm plants). Disease severity was assessed after 7, 14, 17, 21 days post (the pathogen) inoculation (dpi), based on a 0–5 arbitrary scale as follows: 0, leaves expressing no wilting; 1, ≤10% of leaves expressing wilting; 2, 11–25% of leaves with wilting; 3, sectorized wilting, 26–49% of leaves expressing wilting associated with chlorosis; 4, pronounced collapse as leaf extended, 50–74% of leaves expressing wilting; 5, whole plant wilted. A mean disease severity index (DSI) was calculated for each treatment from the score of 30 plants (three replicates of 10 plants for each treatment) in experiment 1 and 14 plants (two replicates of 7 plants per treatment) in experiment 2 using the formula [32]: $DSI (\%) = [(\sum \text{rating no.} \times \text{no. of plants in rating}) \times 100\%] / (\text{total no. of plants} \times \text{highest rating})$.

Measurement of shoot fresh and dry weight

Shoot fresh weight from four different ten-week plants per treatment was weighed, then dried in a hot-air oven at 70 °C for 2 days to have their dry weight.

Assessment of AMF root colonization

After 10 weeks of growth, root samples were collected, then cleaned and stained according to the method using ink and vinegar [41]. The percentage of mycorrhizal colonization was assessed by the gridline intersection method [13]. Briefly, the root sample cut into 1 cm root segments was dispersed randomly in a square Petri dish (10.2 × 10.2 cm) with gridlines on the dish bottom. Horizontal and vertical gridlines were observed under a stereomicroscope at ×100 magnification to record the absence or presence of AMF infection at each point where the root segments intersected a line. Four replications per a root sample were implemented, and four root samples from four plants each treatment were used.

Statistical analysis

Statistical analysis was carried out using SAS 9.1 statistical package. Our data were analysed by one-way analysis of variance (ANOVA) followed by Duncan posthoc test at P<0.05.

RESULTS

Rhizophagus irregularis induces plant resistance against Cmm effectively among AMF isolates tested

After 10 weeks of growth, mycorrhizal colonization rate among AM treatments was significantly different (Fig. 1A) while no substantial differences in plant growth responses of all treatments could be observed (data not shown). The highest colonization level (64.5%) was gained by *Rhizophagus irregularis* (MUCL43194), the most widespread and most frequently studied AM fungal species while the lowest colonization was found by *Gigaspora margarita* (37.4%).

In addition to different responses to mycorrhizal inoculation, three levels of responses to the bacterial disease were also recognized at 17 and 21 dpi although no significant differences in DSI among treatments were found at 7 and 14 dpi (Fig. 1B). Tomato plants inoculated with *Rhizophagus irregularis* showed both the highest colonization and the highest induced resistance to Cmm after 21 days of bacterial infection, while the effect of other isolates (*Funnelformis mosseae*, *Gigaspora margarita* and *Claroideoglomus claroideum*) on the mycorrhizal colonization and the induced resistance were intermediate and high, respectively. Surprisingly, plants inoculated with *Gigaspora margarita* showed the lower colonization than other tested isolates, whereas the high resistance to Cmm in that treatment (*Gigaspora margarita*) was observed. No significant differences in plant biomass of all treatments were found but Cmm resistance induced by *Funnelformis mosseae*, *Gigaspora margarita*, *Claroideoglomus claroideum*, particularly *Rhizophagus irregularis* was observed, suggesting that the MIR was not related to plant growth enhanced by AMF.

Rhizophagus irregularis induced resistance is dependent on ethylene

In order to explore the involvement of ET in mycorrhiza-induced resistance, we used Never ripe (Nr), whose one member of the ET receptor gene family is mutated, resulting in ET insensitivity in tomato plants [20] and its corresponding background (Pearson), while *Rhizophagus irregularis* was chosen as AMF inoculation based on the result of our experiment 1. Ri-induced resistance was also observed in the background plants inoculated by Ri at 7, 14, 17, 21 dpi, confirming the result of our experiment 1 (Fig. 2). In addition, ET insensitivity limited disease development of Cmm due to the fact that DSI of Nr plants was considerably lower than that of the Pearson background during three weeks of Cmm infection. Remarkably, insensitivity of ET in Nr plants colonized with Ri eliminated the MIR against Cmm, when its DSI was similar to that of Pearson plants without Ri inoculation over the course of Cmm infection. These suggest that ET plays a key role in Ri-induced resistance against Cmm.

Noticeably, AM colonization failed to increase shoot fresh and dry weight in plants in our experimental conditions, where no remarkable differences in shoot fresh and dry weight between Pearson and Pearson + Ri, Nr and Nr + Ri were detected (Table 1).

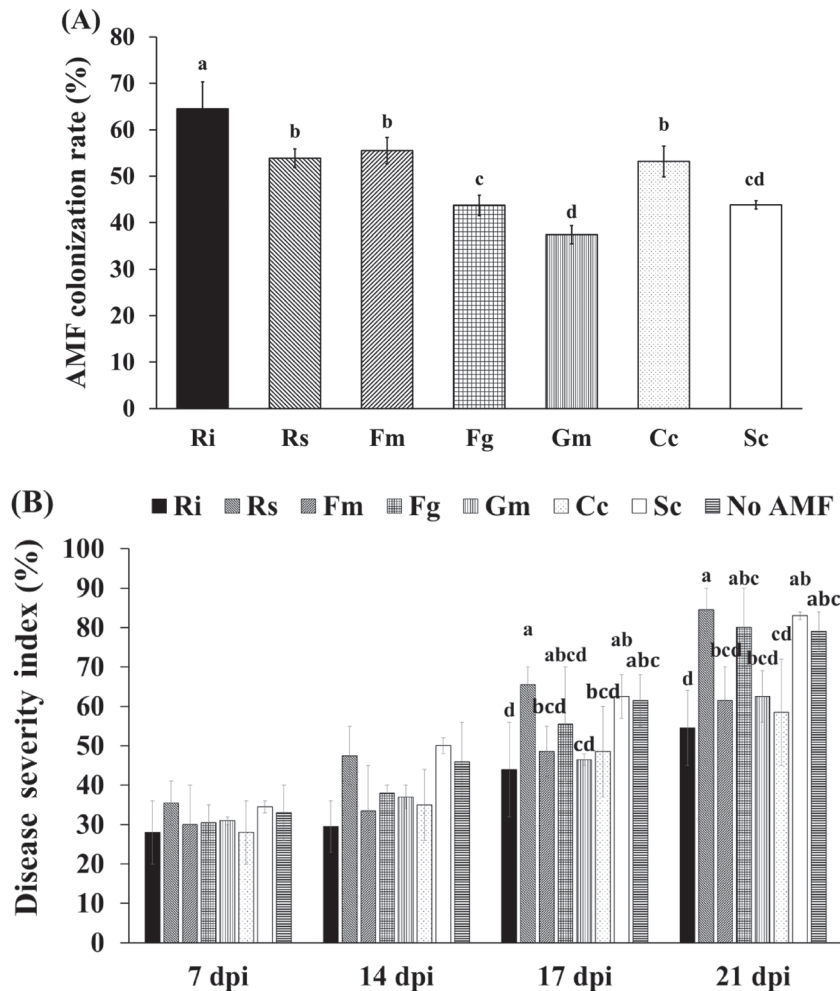


Fig. 1. Arbuscular mycorrhizal fungi (AMF) colonization rate (A) after 10 weeks of growth and disease severity index (DSI) (B) in arbuscular mycorrhizal (AM) and non-AM tomato plants at 7, 14, 17, 21 days post inoculation of Cmm (dpi). Ri, *Rizophagus irregularis* MUCL 43194; Rs, *Rhizophagus* sp. MUCL 43204; Fm, *Funneliformis mosseae* BEG 12; Fg, *Funneliformis geosporum* BEG 11; Gm, *Gigaspora margarita* BEG 34; Cc, *Claroideoglomus claroideum* BEG 23; Sc, *Septoglomus constrictum*. Bars present means \pm Standard Error. No significant differences in DSI among treatments at 7 and 14 dpi. Different letters denote significant differences in DSI among treatments at 17 and 21 dpi

Cmm significantly decreased shoot fresh and dry weight in Nr mutant and its background but the more pronounced reduction in shoot dry weight was found in the treatment Nr+Ri+Cmm. Interestingly, AM colonization rate in Nr+Ri increased, as compared to Pearson+Ri, whilst the percentage was most severely decreased in Nr+Ri+Cmm.

Table 1

Shoot fresh and dry weight, AM colonization rate in Never ripe (Nr) tomato mutant and its background Pearson with or without *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) infection

Treatment	Shoot fresh weight (g)	Shoot dry weight (g)	AM colonization (%)
Pearson	26.35 ± 2.6 ^{ab}	1.97 ± 0.1 ^a	0
Pearson + Cmm	13.95 ± 1.7 ^b	0.93 ± 0.2 ^b	0
Pearson + Ri	28.76 ± 1.9 ^a	2.10 ± 0.2 ^a	54.0 ± 0.7 ^b
Pearson + Ri + Cmm	14.40 ± 2.2 ^b	1.04 ± 0.2 ^b	52.5 ± 1.1 ^b
Nr	20.74 ± 1.5 ^A	1.44 ± 0.2 ^A	0
Nr + Cmm	13.69 ± 1.0 ^B	1.09 ± 0.1 ^B	0
Nr + Ri	20.11 ± 1.4 ^A	1.52 ± 0.1 ^A	65.1 ± 0.3 ^b
Nr + Ri + Cmm	10.06 ± 1.3 ^B	0.75 ± 0.1 ^C	38.5 ± 2.1 ^c

AM – arbuscular mycorrhizal; Ri – *Rhizophagus irregularis*. Parameters were shown as the mean value of four replicates ± Standard Error. Different regular and capital letters each column express significant differences in shoot fresh and dry weight of the background Pearson and Nr mutant, respectively. Different letters in AM colonization column indicate significant differences among plants pretreated by Ri.

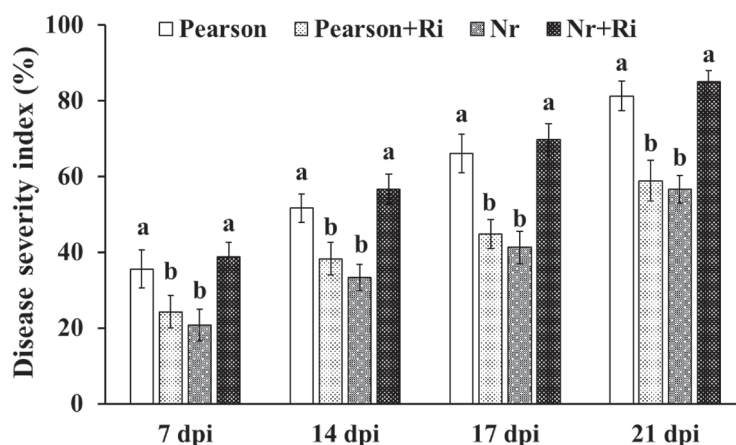


Fig. 2. Disease severity index (DSI) of arbuscular mycorrhizal (AM) and non-AM tomato plants at 7, 14, 17, 21 days post inoculation (dpi) of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in ethylene insensitive mutant (Nr) and its wild-type (Pearson). Ri, *Rhizophagus irregularis* MUCL 43194. Bars present means ± Standard Error. Different letters denote significant differences in DSI among treatments at 7, 14, 17 and 21 dpi, respectively

DISCUSSION

Different AM colonization rate of the isolates found in our results reflects various AM colonization strategies. *Rhizophagus* isolates, high colonization in the present study, produce an extensive hyphal network providing a competitive advantage over other isolates [37], whereas *Gigaspora* sp. shows a lower capacity in that process as we also recognized it, moreover, its re-establishment of mycelium through their anastomoses also offers a smaller capacity to colonize the roots compared to other species [31]. Other tested strains in our work, namely *Rhizophagus* sp. (MUCL 43204), *Claroideoglomus claroideum* (BEG 23) and *Funneliformis mosseae* (BEG 12) had an intermediate level of colonization, confirming that tomato is intermediate in mycorrhizal dependency [28].

It is known that AM symbiosis could enhance plant resistance against a wide range of viral, bacterial and fungal pathogens [10, 12, 21, 23, 38], but our report is the only one regarding the bacterial disease caused by Cmm. The present study showed that Ri induced the most effective systemic resistance against Cmm among seven AMF isolates tested. Different mechanisms are proposed to interpret the role of AMF in plant protection. During mycorrhization, AMF-induced modulation of plant defence responses takes place to achieve a functional symbiosis, leading to activating host immunity locally as well as systemically, called primed state of the plant. This state allows the plant to trigger defence responses more quickly and effectively upon being attacked by potential enemies [17]. Furthermore, a remarkable transcriptional reprogramming, significant alterations in the hormonal balance, primary and secondary metabolisms occur in the host plants during mycorrhization [17, 22]. Transcriptional changes in both roots and shoots in *Medicago truncatula* plants inoculated by *Rhizophagus irregularis* (syn. *Glomus intraradices*), *Gigaspora gigantea*, *Glomus versiforme* resulted in an increased resistance to the shoot pathogen *Xanthomonas campestris* pv. *alfalfae* [21]. Even, underground common mycorrhizal networks of tomato plants pretreated by *Funneliformis mosseae* (syn. *Glomus mosseae*) induced resistance of neighbours against *Alternaria solani* [39]. More recently, AMF-primed resistance to *Alternaria solani* has been proved in tomato plants treated with *Funneliformis mosseae* when they showed enhanced expressions of important defense genes and higher activities of defense-related enzymes as compared to non-AM plants [38].

It is believed that AMF can enhance its host plant nutrient and growth but improved mycorrhizal plant growth was not observed in both of our experiments, perhaps due to the fact that plants were cultivated in pots under suboptimal conditions. Thus, the protective role of AMF was associated with mechanisms other than a better plant fitness, most probably linked to plant defenses.

AM colonization rate in ET insensitivity mutant was increased in relation to the Pearson background, which is in line with the results of several studies revealing that ET has detrimental effects on mycorrhizal development in the symbiosis [11, 33]. It should be noted that the colonization was most profoundly decreased in Never ripe plants with Ri and Cmm inoculation, suggesting that Cmm negatively affected AM

development in ET-insensitive mutant. This may be owing to the fact that Cmm weakened plant fitness, leading to decreasing photosynthate source for the mycorrhizal symbiont, and/or Cmm activated the plant defense system that consequently inhibited the mycorrhizal development. Similarly, the aboveground pathogen *Colletotrichum gloeosporioides* decreased belowground AM colonization in *Phaseolus vulgaris* due to its activation of plant defense responses [5].

ET, a main component of plant defense signals, is generated during microbe–plant interactions. ET can act as a crucial regulator of plant immunity [6, 40]. Our analyzed results indicated that on the one hand, impaired perception of ET decreased significantly the development of wilt symptoms caused by Cmm. This is in accordance with earlier studies revealing that ET in the host plant is crucial in the regulation of the susceptibility to Cmm in tomato plants [4, 34, 35]. On the other hand, ET is required for MIR against Cmm due to the fact that Nr plants pretreated by Ri did not induce resistance against this bacterial pathogen. Previous reports have emphasized a necessary role of Jasmonate-regulated pathway in MIR against different pathogens in several plants [25–27, 30, 38]. Nonetheless, it is worth noting that there is little information about the involvement of ET signaling pathway in MIR. Our result is the first observation of MIR against Cmm mediated by ET. Numerous studies demonstrated that together with Jasmonate, ET plays a central role in control of induced resistance by various beneficial microbes such as *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61 [43] in tomato plants, *Trichoderma harzianum* T39 [19], *Pseudomonas fluorescens* Q2-87 [42], *Pseudomonas protegens* CHA0 [16], *Penicillium* sp. GP16-2 [15] in *Arabidopsis*. In fact, ET is able to stimulate production of distinct pathogenesis-related (PR) proteins or phytoalexins derived from the phenylpropanoid pathway leading to rigidification of cell walls in a wide range of plant species [1, 3], thus enhancing plant resistance against pathogens. Additionally, JA- and ET-signaling pathway often operate synergistically to induce the effector genes of induced defense responses [8, 29, 36].

In conclusion, our results demonstrate that AM colonization could induce systemic plant resistance against Cmm; however, its efficiency depends on specific isolate of AMF, and ET signaling pathway is required for MIR against Cmm. Further studies are necessary to elucidate the mechanisms involved in the induced resistance to Cmm.

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